Characterization of glutathione S-transferases from the pinewood nematode, *Bursaphelenchus xylophilus*

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Summary

Glutathione S-transferases (GST) are enzymes involved in detoxification metabolism and have been found in several plant-parasitic nematodes. In a previous study, we identified two secreted GSTs expressed in the dorsal pharyngeal gland cell of the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, which are upregulated post infection of the host. This study aimed to examine the functional role of GSTs in *B. xylophilus* biology. We analysed the expression profiles of all predicted GSTs in the nematode genome. The results showed that all nematode GSTs belong to the kappa and cytosolic subfamilies (which includes zeta and sigma classes) and more than 50% of these enzymes are upregulated post infection of the host. A small percentage of these are potentially secreted and none are downregulated post-infection of the host. One secreted protein, expressed in the dorsal pharyngeal gland cell (BUX.s00647.112), was confirmed as a functional GST and is within a cluster of GST sequences that show the highest expression fold change in early infection. We showed that this enzyme has a protective activity that may involve host defences, namely in the presence of terpenoid coumpounds (α-pinene, β-pinene and limonene) and peroxide products. These results suggest that GSTs secreted into the host participate in the detoxification of host-derived defence compounds and enable successful parasitism.

Keywords

Detoxification metabolism; pinewood nematode; effectors; terpenoid compounds

Glutathione S-transferases (GST, EC 2.5.1.18) are enzymes involved in detoxification metabolism and are present in a range of different organisms including bacteria, plants and animals. The main function of this large family of enzymes is the detoxification of potentially damaging endogenous stress products and exogenous xenobiotic compounds and also an important role in drug metabolism. This is achieved by the ability to catalyse the conjugation of the reduced form of glutathione (GSH) to potential toxins in order to increase their solubility and thus enable them to be metabolised or excreted from the host (Brophy and Pritchard, 1994; Campbell et al., 2001; Torres-Rivera and Landa, 2008; Matouskova et al., 2016). GST does not act directly on reactive oxygen species (ROS), but on the oxidized products of their activity, including lipid hydroperoxides and reactive carbonyls (Torres-Rivera and Landa, 2008). In parasitic species GST is an important detoxification enzyme, especially in helminths where GSTs provide initial defence against oxidative damage and protect the worm from the host immune response, as well as acting as drug-binding proteins (Precious and Barrett, 1989; Brophy and Barrett, 1990; Brophy and Pritchard, 1994; Matouskova et al., 2016). Therefore, the roles of these enzymes in the host-parasite interaction have been studied extensively. Recent studies on GSTs from animal parasitic helminths showed that sigma-GSTs have prostaglandin synthase activity, and bind to toxins to a suppression of the host immune response to the benefit of the parasite (van Rossum et al., 2004; Dowling et al., 2010; LaCourse et al., 2012). In addition, analysis of the secretome of the animal parasitic trematode *Fasciola hepatica*, revealed sigma class-GST in extracellular vesicules that are deployed during parasitism (Cwiklinski et al., 2015). In the plant parasitic nematode *Meloidogyne incognita*, one GST has been identified as being secreted from the pharyngeal gland cells (*Mi-gst*-1) and plays an important role in the interaction with the host as evidenced by the fact that silencing of this gene by RNAi leads to a reduction in parasitism. This GST may protect the nematode against host derived ROS or may modulate plant responses that are triggered by nematode attack (Dubreuil et al., 2007).

Parasitic helminths contain several forms of GSTs which can be grouped in subfamilies on the basis of their subcellular location: kappa (mitochondrial), microsomal and cytosolic (soluble GSTs from the mu, alpha, pi, theta, sigma, zeta and omega classes) (Frova, 2006; Torres-Rivera and Landa, 2008). Several GSTs have been identified in migratory plant-parasitic nematodes (PPN), including *Bursaphelenchus xylophilus*, *Ditylenchus africanus*, *Pratylenchus coffeae*, *Radopholus similis* and from the sedentary species *Meloidogyne* spp. and *Globodera pallida* (Bellafiore et al., 2008; Dubreuil et al., 2007; Haegeman et al., 2009; Haegeman et al., 2011; Kikuchi et al., 2011; Cotton et al, 2014; Espada et al., 2016; Jacob et al., 2008). A total of 65 potential GSTs were predicted from the genome of *B.*

xylophilus, a similar number to that in *C. elegans,* but higher than seen in other PPN (Kikuchi et al., 2011).

When the pinewood nematode (PWN), *B. xylophilus*, infects a tree it triggers several physical and chemical alterations leading to the symptoms of pine wilt disease (PWD). Kuroda et al., (1991) hypothesised a mechanism of cavitation, in which terpenoids synthesised in xylem ray cells induce cavitation and embolisms in tracheids leading to failure of water transport. Previous studies have shown that levels of plant terpenes in *P. thunbergii*, particularly α-pinene and β-pinene, increase when the tree is infected by *B. xylophilus* (Fukuda et al., 1997; Kuroda et al., 1991; Kuroda, 1991). However, a recent study examining infection of plant material maintained in tissue culture, suggested that terpenoid compounds do not significantly increase after infection with PWN although levels were maintained after infection, with α-pinene making up between 26%-32% of total terpenoid content and β-pinene between 34%-47% (Faria et al., 2015). Several of these compounds have nematicidal activity, although no study has been made in *B. xylophilus*. Chemical compounds including terpenoids have been tested against filarial nematode GST and one study showed that α-pinene has an inhibitory effect on the nematode GST (Azeez et al., 2012).

In a previous study, we identified two secreted glutathione S-transferases that were upregulated in an early stage of infection and which are expressed in the dorsal pharyngeal gland cell (Espada et al, 2016). It was suggested that these enzymes could be involved in detoxification of plant endogenous compounds, helping *B. xylophilus* to overcome host defences. Here we demonstrate that at least one of these is a functional GST and that the presence of this enzyme provides protection against stresses likely to be encountered during infection of the host tree. We show that biochemically active GST is secreted by nematodes. In addition, we examine the global changes in expression of *B. xylophilus* GSTs upon infection of the host.

Materials and methods

Phylogenetic analysis of GST sequences

Potential GST-encoding sequences were identified using the previous data from Kikuchi et al. (2011) and by BLASTP searching the gene calls from the *B. xylophilus* genome against the NR database (cutoff $1e^{-5}$). Any sequences for which at least one of the top three hits included the expression "glutathione S-transferase", were selected for further analysis. This analysis was performed using BLAST+ wrappers for Galaxy (v0.1.01) (Cock et al., 2015). The expression levels of the transcripts at various life stages were predicted from RNAseq data generated in a previous study (Espada et al.,

2016) and log2 of the fold change for each gene was calculated. For all the predicted GSTs the subfamilies and protein domains were identified using InterProScan 5 (Jones et al., 2014) (http://www.ebi.ac.uk/interpro/search/sequence-search). Secreted GSTs were predicted based on the presence of signal peptide as predicted by SignalP (v3.0) (Petersen et al., 2011) and the absence of a transmembrane domain. All the alignments of the full-length protein sequences were performed with the software SeaView (Gouy et al., 2010). The Maximum-likelihood phylogenetic tree was generated by PhyML (in SeaView) from the alignment of the sequences (protein distance measure: Jukes-cantor; aLRT SH-like for branch supporting). The phylogenetic tree was edited in FigTree (v1.4.0) (http://tree.bio.ed.ac.uk/software/figtree/). A neighbour-joining phylogenetic tree was generated in the software CLC Sequence Viewer (v7.6.1) (protein distance measure: Jukes-cantor; one thousand replicates for bootstrap for branch supporting).

Biological material

Nematodes were cultured on *Botrytis cinerea* and harvested using a Baermann funnel as previously described (Espada et al., 2016). Secreted proteins were collected as described in Kikuchi et al., (2004). Briefly, mixed life stage nematodes were collected in a 15ml-tube, by centrifugation at 2844g for 15 minutes, suspended in 1ml M9 buffer and incubated for 2 days at 18C. After this time, the sample was centrifuged at 2844g to pellet the nematodes, the supernatant containing secreted proteins was collected and stored in aliquots at -80^{o}C until used in enzyme assays.

Cloning in expression vector and protein purification

The primers to amplify the full-length of one of the *B. xylophilus* GSTs shown to be expressed in the dorsal pharyngeal gland cells (BUX.00647.112) were designed from the cDNA sequence lacking the signal peptide (as predicted by SignalP 3.0). The gene specific primers included the Kozak sequence (ACCATG) in the forward primer (5'ACCATGTTAGAGCTGTATTATTTCAACGAGAAG) and a Stop codon (TGA) in the reverse primer (5'TCATTGAGTGGCATTGAAATAATTGTAAATCG). The full length gene was amplified using KOD Hot Start proof-reading DNA polymerase and purified using the QIAquick gel extraction Kit (Qiagen). The gene was cloned into the pCR8 TOPO vector and transformed in one shot TOP10 competent cells following the manufacturer's instructions (Invitrogen). The recombinant clones were screened by colony PCR and one clone was confirmed by sequencing. Purified entry plasmid (approximately 140ng) was transferred to the destination vector pJC40 (a 10xHis-tag N-terminus fusion vector) using the LR cloning kit following the manufacturer's instructions (Invitrogen). The cloning reaction was transformed into BL21(DE3) chemical competent cells. Positive transformants (construct pJC40+00647.112) were analysed by colony PCR and confirmed by

sequencing. The His-tagged protein was induced by adding 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside) to a bacterial culture grown from a single colony in 10ml LB with 100µg/ml ampicillin, at 37C until the concentration reached an OD_{600} of 0.6. The protein was then purified using Ni-NTA resin columns (Ni-NTA Spin kit, Qiagen) following the manufacturer's protocol.

Resistance test in BL21(DE3) cells

To induce expression of the recombinant protein, a single colony was grown in 45ml LB and 100µg/ml of ampicillin, at 37C with agitation, until the concentration reached an OD_{600} of 0.6. At this point 100µl aliquots of the bacterial suspension were placed in new sterile 15ml-tubes containing 4ml LB and to which the terpenes (limonene, (+) and (-)-α-pinene, (-)-β-pinene) or hydrogen peroxide were added. For each treatment, two different concentrations were tested and two replicates were used: 0.5% and 1% for limonene, (+) and (-)-α-pinene, (-)-β-pinene (Sigma-Aldrich); 1% and 3% for hydrogen peroxide. Protein expression was induced in the remaining bacterial suspension by adding 0.5mM IPTG and incubating at 37C, with agitation, for 2 hours. After this time the terpenoid and hydrogen peroxide treatments were repeated using 100 µl aliquots of the bacterial suspension as described above. The respective control tubes were also grown in the same conditions. All the treatments were subsequently grown overnight at 37C with agitation. The OD_{600} was measured for all treatments in a spectrophotometer (Spekol 1500, Analytik Jena). The results were analysed with an ANOVA test using the statistical software GenStat (version 17th; VSN International, 2012).

Western Blotting

Aliquots of the bacterial cells from test described above were used in a Western-blot using an antibody against a poly-histidine tag (Sigma-Aldrich) to demonstrate the presence of the recombinant protein in the assay. The bacterial extracts were heated at 90C for 10 minutes in NuPage LDS sample buffer (Invitrogen). The proteins were separated on a 4-12% NuPage Bis-Tris gel and transferred to nitrocellulose membrane (GE Healthcare). Immuno-detection of the protein was performed using anti-His antibody (Sigma) at 1:5,000 dilution as primary antibody and detected using a secondary antibody conjugated to peroxidase (α-mouse IgGxHRP at 1:50,000) (Sigma) by chemiluminescence using the Pierce Supersignal West Pico kit (Thermo-Scientific).

Enzyme assay

The Glutathione-S-transferase assay kit (Sigma-Aldrich) was used with 1-chloro-2,4-dinitrobenzene (CDNB) as the standard substrate to test activity of recombinant protein and activity present in collected

secretions. All assays were replicated three times. A solution containing 2mM reduced L-glutathione and 1mM CNDB in Dulbecco's phosphate buffered saline was prepared and used within an hour of preparation. 50 µl aliquots of this solution were mixed with 1µl of control GST enzyme or with test enzyme preparations and transferred to a quartz cuvette. Absorbance was measured at 340nm, each 30 seconds over a period of 5 minutes, after a lag time of 1 minute, following the manufacturer's procedure. GST activity was calculated for each sample as described by the kit manufacturer.

Results and Discussion

Global analysis of B. xylophilus *GST expression profiles*

An analysis of GSTs performed as part of the *B. xylophilus* genome project (Kikuchi et al*.,* 2011) identified 65 potential GSTs. Our BLASTP based analysis of the *B. xylophilus* genome revealed that five more sequences, which could encode proteins similar to GSTs, were present (Figure 1). Analysis of the protein domains present in each sequence confirmed all the protein sequences as GSTs, as described in Supplementary Table 1. The majority of these sequences have a thioredoxin-like fold domain (IPR012336) followed by glutathione S-transferase N-terminal and C-terminal domains, both of which are features of cytosolic subfamily (reviewed in Frova, 2006). Five sequences contained domains similar to maleylacetoacetate isomerase (IPR005955), which is a feature of the zeta class of GSTs. The other 4 sequences were identified as kappa subfamily GSTs, due to the presence of the DSBA-like thioredoxin domain (IPR01853) (a feature of the HCCA isomerase/GST kappa family – IPR01440) (Frova, 2006).

Six of the GST sequences have a predicted signal peptide, suggesting a role in detoxification of extracellular compounds, including host derived toxins (Figure 1). These potentially secreted proteins included the two sequences (BUX.s00647.112 and BUX.s01254.333) that were previously identified as being expressed in the pharyngeal gland cells (Espada et al., 2016). Next we used our previously described RNAseq dataset to examine global changes in expression profiles of the GST sequences, by using log2 of the fold changes. This showed that 42 of the GST sequences are upregulated in nematodes after infection of trees as compared to nematodes grown on fungi (Figure 1), including four of those sequences with a signal peptide. None of the secreted GSTs were downregulated after infection. The maximum-likelihood phylogenetic analysis (Figure 1) of the *B. xylophilus* GSTs and sequences from other nematodes showed that the pharyngeal gland cell sequences clustered with other sequences upregulated after infection. One (BUX s01254.333) formed a cluster with other secreted and upregulated protein while the other (BUX s00467.112) clustered with another secreted protein and two other upregulated proteins. This cluster includes the sequences that show the highest increases in expression during the infection of the host. Neither the pharyngeal gland cell GSTs, nor the secreted

GSTs formed a single cluster (although the secreted GSTs were present as pairs in three clusters). These clusters were consistent in a neighbour-joining phylogenetic analysis (Supplementary figure 1). These data suggest that a range of the GSTs present in *B. xylophilus* have been recruited independently to play a role in protection against host derived toxins and that the range of secreted GSTs has not evolved as a result of duplication of a single secreted ancestor.

Enzymatic and protective activity of GSTs involved in the host-parasite interaction

We next examined the biochemical activity of one of the pharyngeal gland cell GST sequences. The recombinant BUX.s00647.112 protein was cloned into an expression vector with an N-terminal His tag and purified from bacterial cell lysate, yielding a protein of approximately 25KDa, in agreement with the size predicted from the amino acid sequence (Figure 2). The recombinant protein had glutathione transferase activity (using CDNB as a substrate) very similar to that observed for the positive control (Table 1). These data confirm that the BUX.s00647.112 protein is a functional GST.

Our previous data showed that several GSTs (including the BUX s00647.112 sequence) are expressed in the pharyngeal gland cells, from where they could be secreted into the host. In addition, a larger scale proteomic analysis of *B. xylophilus* secreted proteins identified several peptides that could be derived from GST sequences (Shinya et al., 2013) further suggesting that GSTs form an important component of the *B. xylophilus* repertoire of secreted proteins. In keeping with this, we were able to detect GST activity (albeit at low levels) in secretions collected from *B. xylophilus* (Table 1). The RNAseq data suggest that it would have been possible to detect higher GST activity in secretions harvested from nematodes extracted from trees but technical limitations prevented us from attempting this analysis.

We next sought to analyse whether the *B. xylophilus* pharyngeal gland cell GST can provide protection against the toxins likely to be encountered by a nematode infecting a pine tree. Testing the function of the GST in pine trees is not possible due to technical limitations. We therefore compared the ability of bacterial cells in which the GST was either induced or not induced to grow in the presence of hydrogen peroxide and several terpenoid compounds. The peroxide was intended to represent the products of ROS while the terpenoids were chosen to mimic toxic compounds likely to be present in an infected pine tree. In the presence of the GST, bacteria showed significantly higher growth in an environment with a (-) and (+)-α-pinene (-)-β-pinene, 0.5% limonene and up to 3% hydrogen peroxide (Figure 3). There were no significant differences in the 1% limonene treatment or in the control (induced vs. non-induced). The difference in growth rate was most apparent in the presence of 0.5% (-)-β-pinene. A Western blot (Figure 2) showed that the recombinant GST was present in all IPTG-induced samples while the non-induced bacterial cells showed no signal in the blot (Figure 2).

These data confirm that *B. xylophilus* secretes functional GST proteins into the host, which may be important for allowing the nematode to overcome host defences. This may be a strategy that is widely used by plant-parasitic nematodes: a secreted GST has been identified from *M. incognita* (*Mi-gst-1*) which has been shown to promote infection of this nematode (Dubreuil et al., 2007) and which is also thought to function by protecting the nematode from host defences. Like the *B.xylophilus* sequence, the *M. incognita* GST is upregulated upon infection and expressed in the pharyngeal gland cells. GSTs also form a significant component of the strategy used by animal-parasitic nematodes to neutralise host defence responses. It is likely that GSTs used for internal metabolic processes have become adapted for a role in the host-parasite interaction in both plant- and animal-parasitic nematodes. Similar adaptation of housekeeping proteins for roles in parasitism in animal and plant parasites has been described previously with peroxiredoxins, glutathione peroxidases and lipid binding proteins all known to be deployed by plant-parasites and animal parasites in order to provide protection from host defences (reviewed by Jasmer et al., 2003). Convergent evolution between animal- and plant-parasitic nematodes is therefore a recurring theme in terms of how they cope with host derived stresses.

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Tables

Table 1 – Glutathione transferase activity results using CDNB as substrate (Blank) in recombinant BUX.s00647.112 protein, *B. xylophilus* protein extract and secretions. The GST protein control was provided on the kit (Sigma). a) Results for the glutathione transferase activity present in the crude extracts of PWN proteins and secretions. b) Results for the enzymatic activity present in the recombinant BUX.s00647.112 protein. Each value is represented by mean ± SD.

Supplementary Table 1 - Protein domains predicted for all 70 putative GSTs from the nematode. Each domain is represented by the InterProScan identification code. For some of the proteins the family was identified.

Figures

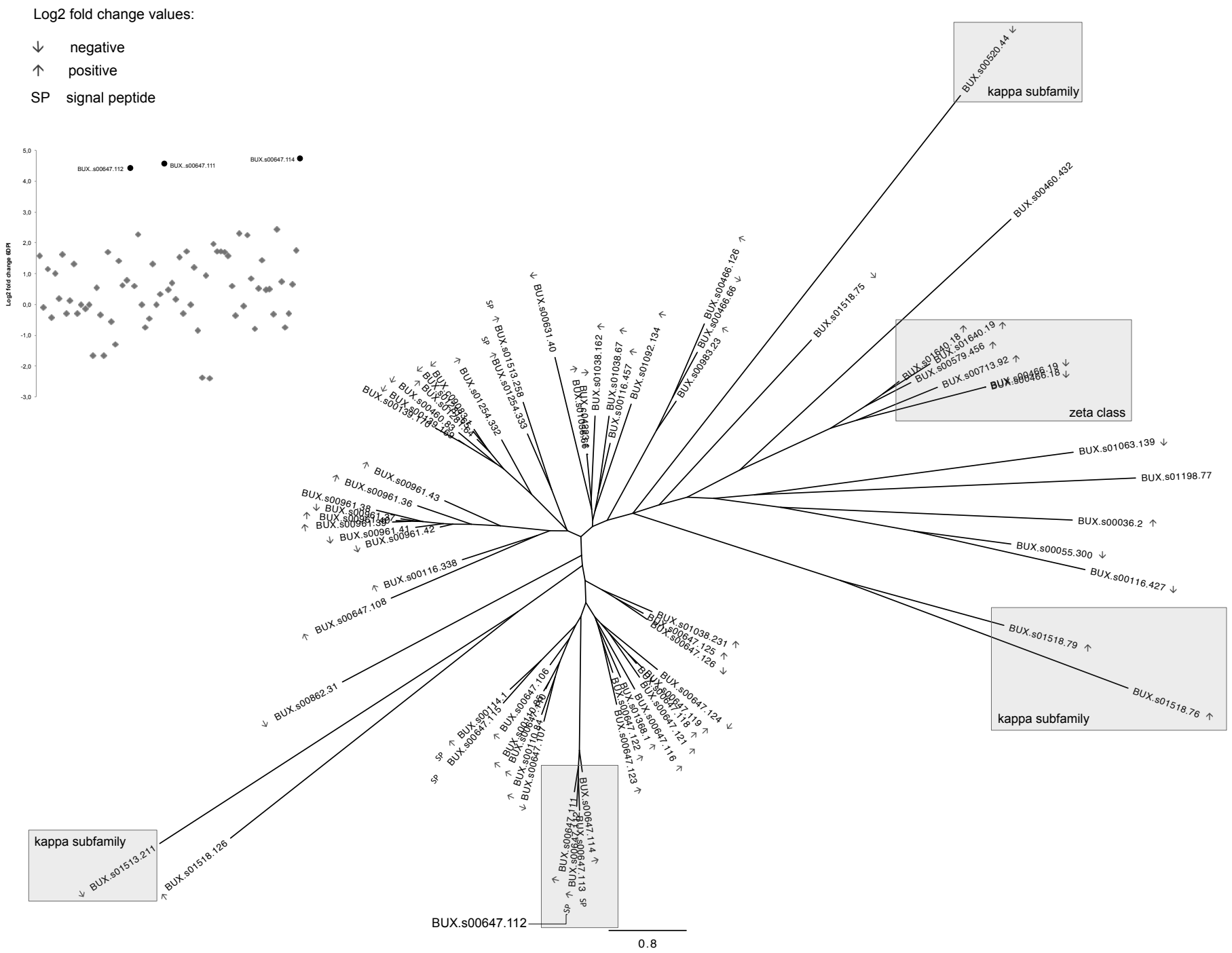
Figure 1 – Maximum-likelihood phylogenetic tree that represents the protein sequence similarity between all 70 PWN predicted GSTs. The GSTs belonging to the kappa subfamily and to the cytosolic zeta class are represented within grey boxes. For each gene the log2 of the fold changes (6 days post infection) values of the expression levels are represented by arrows. The highest log2 fold change values belong to the genes BUX.s00647.112, BUX.s00647.111, BUX.s00647.114 that cluster together, represented within a grey box. The dot plot on the top left of the figure is a representative chart of the expression values of all genes. SP represents the presence of a signal peptide.

Figure 2 – The results of the immuno-detection of anti-Histag on the recombinant BUX.s00647.112 protein resistance assays. On the right, the Ponceau Red staining and on the left the results of the blot detected by chemilumencence. M: protein ladder (GeneRuler, Thermofisher).

Figure 3 – Resistance test in BL21(DE3) cells. Induced vs. non-induced BUX.s00647.112 protein using different pine terpenoid compounds and different concentrations of each (X axis). The values in the Y

axis correspond to values of absorbance (OD_{600}). The LB media was used to grow the bacteria. Protein expression was induced with 0.5mM IPTG (see material and methods). The significant differences between induced and non-induced treatments were analysed by ANOVA statistical test and the results are signed with * for p-value<0.05 and ** for p-value<0.01.

Supplementary Figure 1- Neighbour-joining phylogenetic tree of all 70 protein sequences from *B. xylophilus*. The highlighted clusters in grey boxes represent the kappa subfamily, the zeta classe and the clusters with the protein of interest (BUX.s00647.112) and the proteins with predicted signal peptide. This tree confirms that the clusters are not and artefact of the maximum-likelihood phylogenetic tree.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↓ negative
↑ positive
SP signal peptide
Log2 fold change 6DPI
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BUX..s00647.111
BUX.s00647.114
BUX.s00520.44
kappa subfamily
BUX.s00460.432
BUX.s01518.75
BUX.s00631.40
BUX.s00466.126
BUX.s00466.66
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BUX.s01513.258
BUX.s01254.332
BUX.s01254.333
BUX.s01640.18
BUX.s01640.19
BUX.s00579.456
BUX.s00713.92
BUX.s00466.19
BUX.s00466.18
zeta class
BUX.s01063.139
BUX.s01198.77
BUX.s00036.2
BUX.s00055.300
BUX.s00116.427
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kappa subfamily
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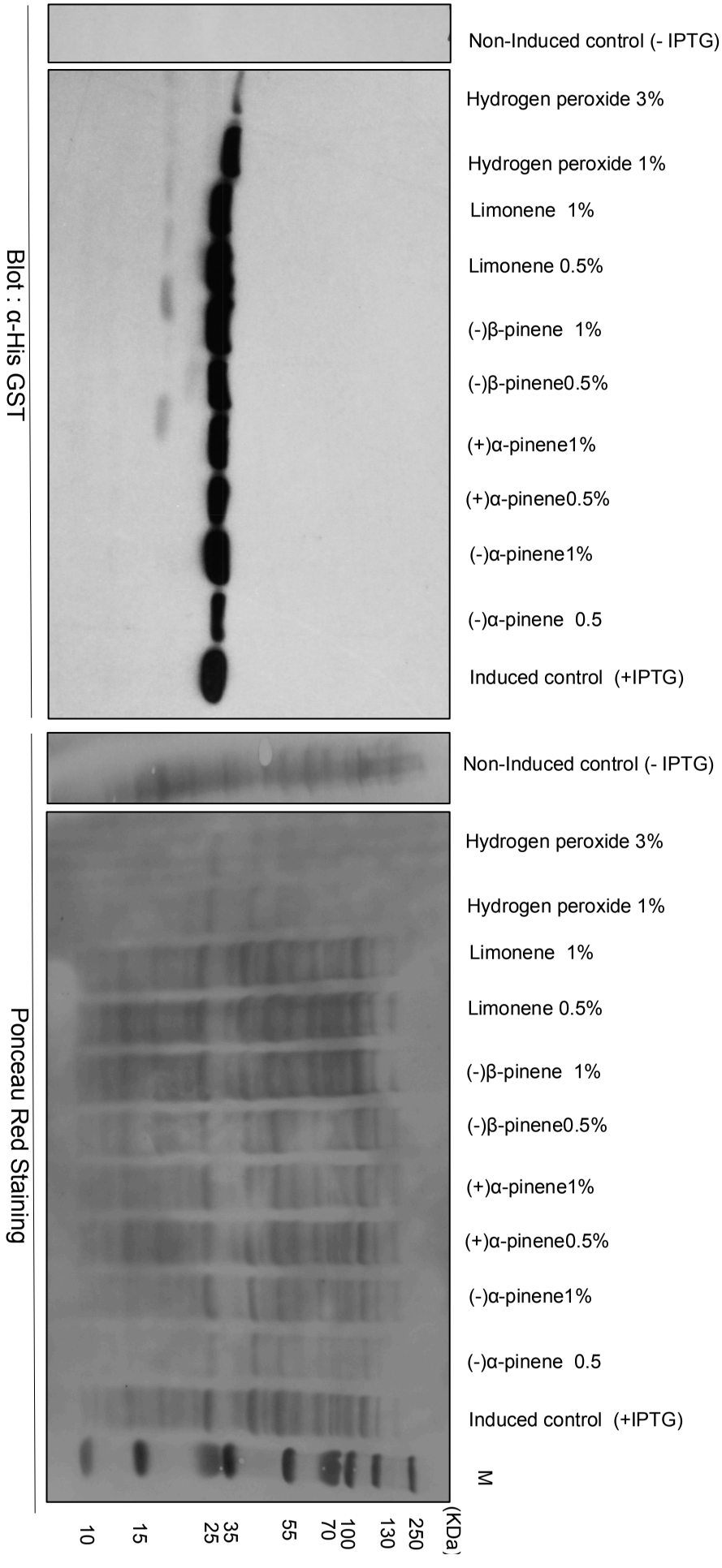
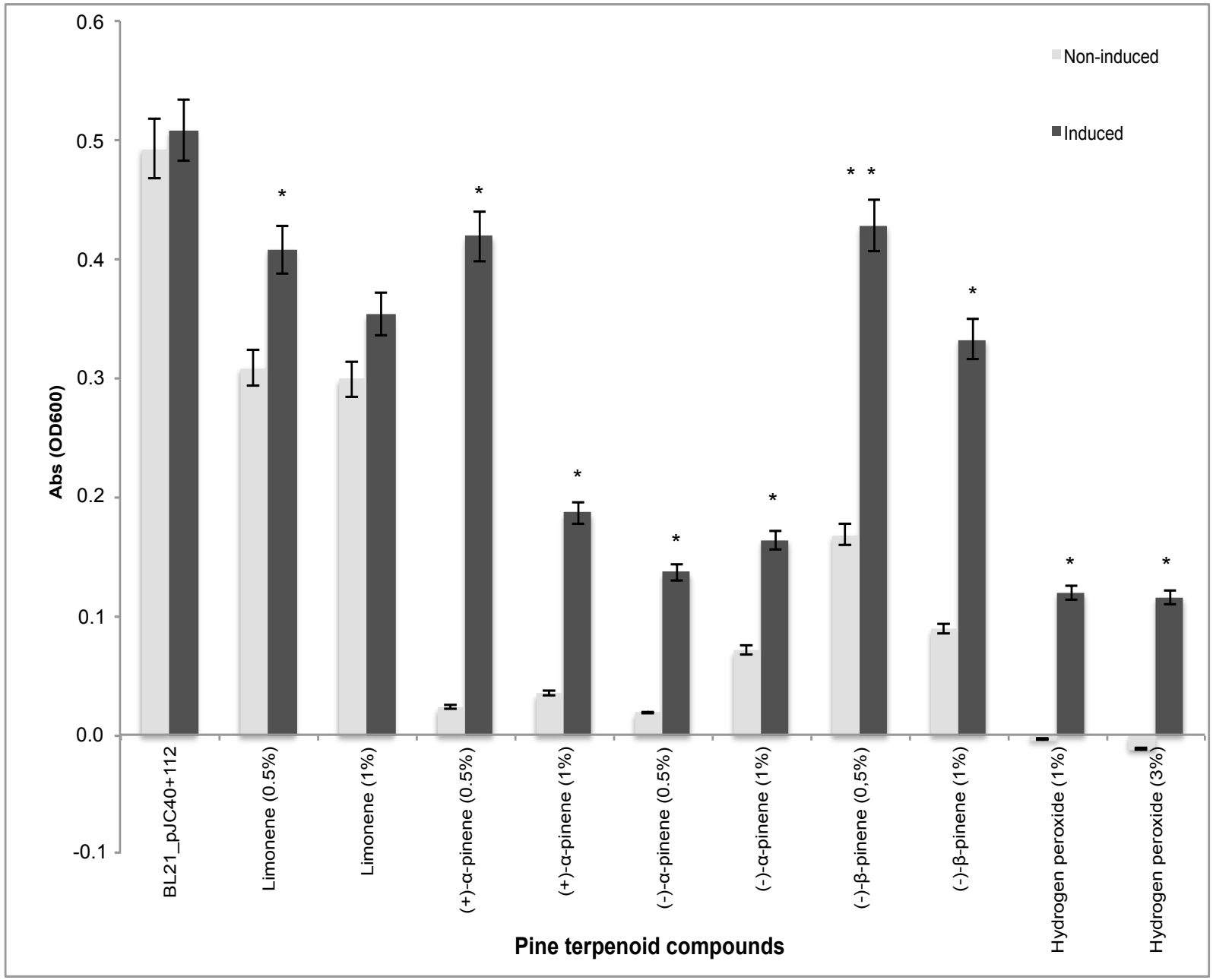
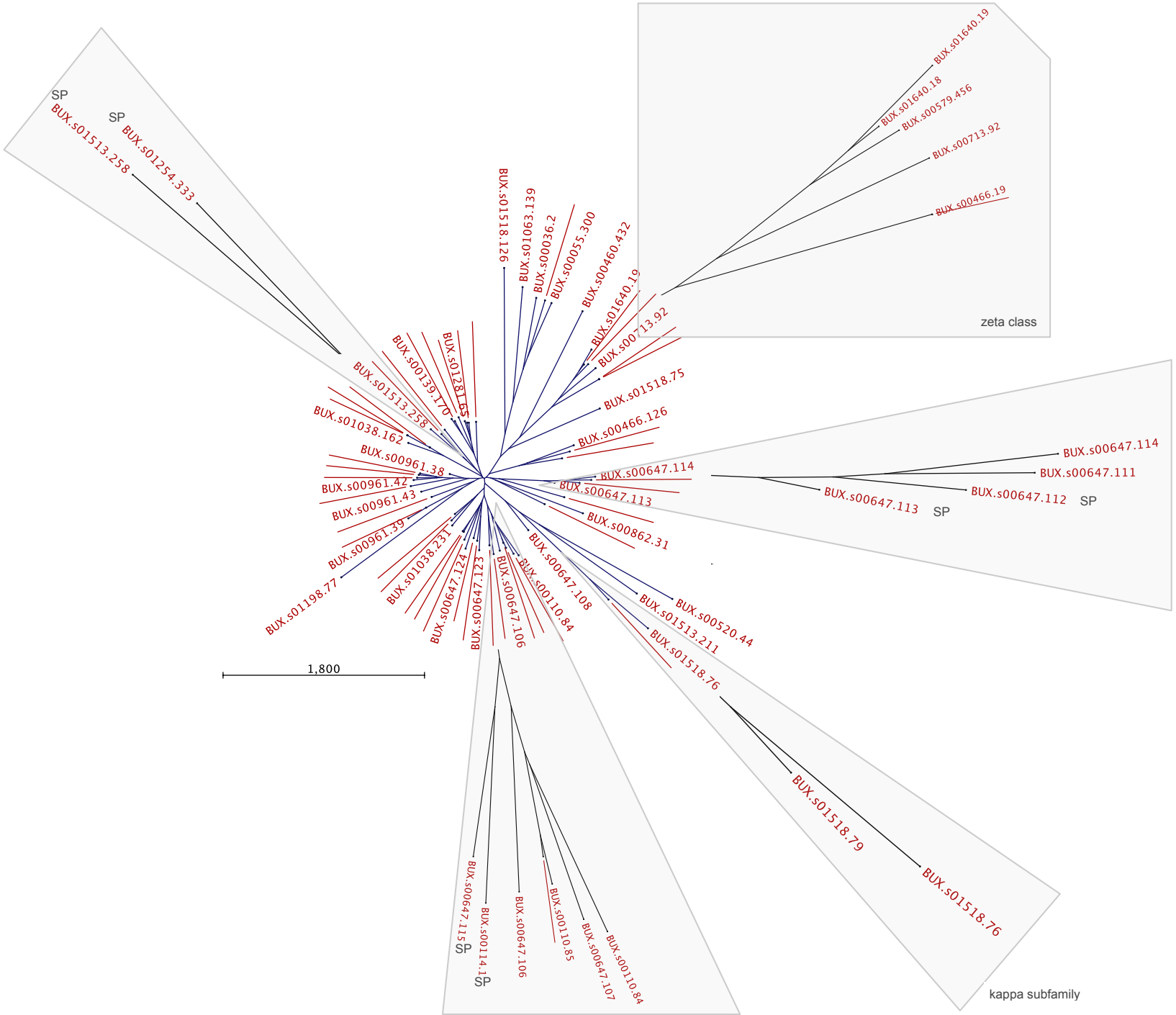
BUX.s01513.211
BUX.s01518.126
kappa subfamily
0.8


Blot : α-His GST
Non-Induced control (- IPTG)
Hydrogen peroxide 3%
Hydrogen peroxide 1%
Limonene 1%
Limonene 0.5%
(-)β-pinene 1%
(-)β-pinene0.5%
(+)α-pinene1%
(+)α-pinene0.5%
(-)α-pinene1%
(-)α-pinene 0.5
Induced control (+IPTG)
Ponceau Red Staining
Non-Induced control (- IPTG)
Hydrogen peroxide 3%
Hydrogen peroxide 1%
Limonene 1%
Limonene 0.5%
(-)β-pinene 1%
(-)β-pinene0.5%
(+)α-pinene1%
(+)α-pinene0.5%
(-)α-pinene1%
(-)α-pinene 0.5
Induced control (+IPTG)
M
10
15
35
25
55
100
70
130
250
(KDa)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
Abs (OD600)
Non-induced
Induced
BL21_pJC40+112
Limonene (0.5%)
Limonene (1%)
(+)-α-pinene (0.5%)
(+)-α-pinene (1%)
(-)-α-pinene (0.5%)
(-)-α-pinene (1%)
(-)-β-pinene (0,5%)
(-)-β-pinene (1%)
Hydrogen peroxide (1%)
Hydrogen peroxide (3%)
Pine terpenoid compounds


SP
SP
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BUX.s01254.333
BUX.s01518.126
BUX.s01063.139
BUX.s00036.2
BUX.s00055.300
BUX.s00460.432
BUX.s01640.19
BUX.s00713.92
BUX.s01518.75
BUX.s00466.126
BUX.s00647.114
BUX.s00647.113
BUX.s00862.31
BUX.s00520.44
BUX.s01513.211
BUX.s01518.76
BUX.s00647.108
BUX.s00110.84
BUX.s00647.106
BUX.s00647.123
BUX.s00647.124
BUX.s01038.231
BUX.s01198.77
BUX.s00961.39
BUX.s00961.43
BUX.s00961.42
BUX.s00961.38
BUX.s01038.162
BUX.s01513.258
BUX.s00139.170
BUX.s01281.155
1,800
BUX.s01640.19
BUX.s01640.18
BUX.s00579.456
BUX.s00713.92
BUX.s00466.19
zeta class
BUX.s00647.114
BUX.s00647.111
BUX.s00647.112
SP
BUX.s00647.113
SP
BUX.s01518.79
BUX.s01518.76
BUX.s01518.76
kappa subfamily
BUX.s00647.115
SP
BUX.s00114.1
SP
BUX.s00647.106
BUX.s00110.85
BUX.s00647.107
BUX.s00110.84

a

Sample (CDNB as substrate)	**GST activity (µmol/ml/min)**
GST (control)	133.7 ± 62.3
B. xylophilus secretions	31.2 ± 1.9
B. xylophilus proteins	37.1 ± 0.2

b

Sample (CDNB as substrate)	**GST activity (nmol/ml/min)**
GST (control)	1509.8 ± 73.4
Recombinant BUX.s00647.112	2096.3 ± 312.5